Diversity of naturally occurring Epstein–Barr virus revealed by nucleotide sequence polymorphism in hypervariable domains in the BamHI K and N subgenomic regions

Dimitris Triantos,1, 2 Alison W. Boulter,1, 3 Jair C. Leao,1, 2 Luca Di Alberti,1, 2 Stephen R. Porter,2 Crispian M. Scully,2 Warren Birnbaum,4 Newell W. Johnson3 and Chong Gee Teo1

1 Virus Reference Division, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK
2 Department of Oral Medicine, Eastman Dental Institute for Oral Health Care Sciences, University of London, 256 Gray’s Inn Road, London WC1X 8LD, UK
3 Department of Oral Medicine and Pathology, King’s College School of Medicine and Dentistry, Caldecot Road, London SE5 9RW, UK
4 Department of Primary Dental Care, King’s College Dental Institute, Caldecot Road, London SE5 9RW, UK

The extent of nucleotide sequence microheterogeneity varies among subgenomic regions of Epstein–Barr virus (EBV). We examined, in EBV-carrying lymphoid cell lines, the extent of polymorphism in EBV DNA fragments amplified from the BamHI E, K, N and Z regions, and then investigated the diversity of the more hypervariable regions in tissues and body fluids. In cell lines, sequence dissimilarities in a genotype-specifying fragment of the EBNA-3C gene varied from 1–4% within each genotype; dissimilarities in the first intron of the BZLF-1 gene were 2% within each genotype. By contrast, dissimilarities in a C-terminal unique domain of the EBNA-1 gene, and in a fragment that encompasses and is upstream of the LMP-1 start codon, varied between 2 and 7% and were not genotype-specific. The sequence diversity in BamHI K and N regions was then examined in tissues and body fluids by single-strand conformation polymorphism (SSCP) analysis and cycle sequencing. Extensive inter-host diversity was observed, whether the host was co-infected by human immunodeficiency virus (HIV) or not. In the oral cavity of HIV-infected patients, inter-compartmental EBV diversity could be demonstrated, even between sites that were anatomically proximate. Studies of BamHI K clones derived from EBV in oral lesions revealed infection by multiple variants. Identification of hypermutable loci within the EBV genome such as those located in the BamHI K and N regions should permit fine discrimination of individual EBV variants.

Introduction

Epstein–Barr virus (EBV) is a significant pathogen and is widespread in human populations (Rickinson & Kieff, 1996). It is also genetically diverse (Gratama & Ernberg, 1995). EBV variants are frequently distinguished according to sequence polymorphism in the open reading frame (ORF) encoding the EBNA-2 antigen, these polymorphisms segregating with those in the ORFs of the EBNA-3A, -3B and -3C antigens (also called 3, 4 and 6), which are tandemly arranged in the BamHI E region. Two EBV types are differentiated in this way, designated EBV-1 and -2, or types A and B. In Western, East Asian and North African populations, the detection rate of type 1 far exceeds that of type 2, while in the general population of equatorial Africa, Papua New Guinea and Alaska, type 2 appears to be as common as type 1 (Gratama & Ernberg, 1995). However, the epidemiology is not clear-cut, as type 2 is frequently found in Western patients who are immunosuppressed or have Hodgkin’s disease (Gratama & Ernberg, 1995), and even in apparently healthy people (Sixbey et al., 1989; Apolloni & Sculley, 1994).

EBV variants may also be distinguished according to the retention or loss of an XhoI restriction site in the LMP-1 gene, or the presence or absence of a 30 bp deletion at the 3’ end of the LMP-1 ORF (Hu et al., 1991). Variants without the XhoI

Author for correspondence: Chong Gee Teo.
Fax +44 181 200 1569. e-mail BPurohit@PHLS.co.uk
restriction site and possessing the 30 bp deletion are positively correlated with nasopharyngeal carcinoma (NPC) and lymphoproliferative disorders (Jenkins & Farrell, 1996). Further differentiation of EBV variants can be based on a BamHI restriction site between the BamHI W1* and H1* fragments, and an additional BamHI site in the BamHI F fragment, giving rise to the C/D and F/f genotypes, respectively (Lung et al., 1990); these genotypes may be geographically restricted (Gratama & Ernberg, 1995). Distinct intra-host EBV genotypes containing either two or three copies of a 29 bp tandem repeat sequence in the first intron of the BZLF-1 gene have also been reported (Chen et al., 1996), with the genotype that bears three copies appearing to infect the epithelium preferentially.

Other than these dichotomies, EBV can be differentiated according to size polymorphisms, depending on the number of internal repeats in the BamHI E, H, I, K and N regions (Lung et al., 1988; Miller et al., 1994; Falk et al., 1995). A variety of natural EBV variants have been discovered, and inter-person transmissions traced (Lung et al., 1991; Cen et al., 1991; Alfieri et al., 1996; Falk et al., 1997). Furthermore, discrete mutations, involving point substitutions and deletions or insertions of single bases or longer stretches in other genomic regions, have also been observed. Potentially, these would allow variants to be discriminated even more finely. Hypervariability has thus been identified in the following regions: the C-terminal domain of the EBNA-1 ORF in the BamHI K region (Wrightham et al., 1995; Snudden et al., 1995; Bhatia et al., 1996; Gutiérrez et al., 1997); the EBNA-2 gene, which is located in the BamHI WYH region (Aitken et al., 1994); several domains in the BamHI N region, including the LMP-1 gene (Miller et al., 1994), the N terminus of the LMP-2A gene (Busson et al., 1995), and an approximately 300 bp region upstream of the LMP-1 start codon (Hu et al., 1991); and the BZLF-1 gene (Packham et al., 1993).

We have studied sequence polymorphisms, by DNA sequencing and single-strand conformation polymorphism (SSCP) assays, in potential hypervariable domains of the BamHI K, N and Z regions in EBV-carrying cell lines, and in tissues and body fluids of hosts who were and were not infected by human immunodeficiency virus (HIV). Possible hypervariability in the type 1/2-specifying locus within the BamHI E region encoding EBNA-3C (Sample et al., 1990) was also examined.

Methods

Cell lines. The EBV-infected lymphoid cell lines B95-8, EB176, EB185, P3HR-1, AW-Ramos, AG876, Namalwa, Daudi and Raji (all obtained from the European Collection of Cell Cultures) were propagated in RPMI 1640 supplemented with 10% bovine calf serum. Lines EB176 and EB185 are derived from peripheral lymphocytes of a chimpanzee and an orangutan, respectively, having been transformed by virus of the B95-8 line (J. Clegg, personal communication). EBV carried by these and the Namalwa, Daudi and Raji cell lines are type 1 viruses. AW-Ramos is a converted cell line, being derived from the Ramos cell line that was infected by EBV from P3HR-1 cells (Andersson & Lindhal, 1976). EBV in the AW-Ramos, P3HR-1 and AG876 lines are type 2 viruses.

Clinical and biopsy specimens. Clinical specimens were obtained from patients known to be infected by HIV and attending outpatient clinics in London, UK and Recife, Brazil. Tongue scrapings were obtained by scraping the lateral border of the tongue with a sterile curette which was then washed with PBS, and particulate matter was pelleted. Whole saliva was collected (after mechanical stimulation by chewing sterile rubber bands), clarified and passed through 0.45 µm filters. To collect parotid saliva, a Lashley cup was applied to Stensen’s duct after stimulation of the tongue with citric acid crystals, after which salivary fluid was passed through 0.45 µm filters. Peripheral blood cells (PBCs) drawn into EDTA-treated Vacutainers were enriched for CD45+ cells by an immunomagnetic bead procedure, performed as recommended by the manufacturer (Dynal). Tissue biopsy specimens were derived from the following tissues: two diagnosed as bronchogenic carcinomas, two squamous cell carcinomas of the skin, one oral ulcer, one oral hairy leukoplakia (OHL), one oral lesion from a patient with Sweet’s syndrome and one NPC. All biopsy specimens were formalin-fixed and paraffin-embedded.

DNA extraction. DNA was extracted from cells and saliva specimens by the Chelex-100 extraction protocol (Ochert et al., 1994), from tongue samples by the proteinase K digestion method, and from blood samples and tissue sections with GeneClean II (BIO 101).

PCR amplification. Primers were selected to allow amplification of the following: by nested PCR, a 433 bp segment of the EBNA-1 ORF in the BamHI K region (Wrightham et al., 1995); by hemi-nested PCR, a 368 bp segment that encompasses and is upstream of the LMP-1 start codon in the BamHI N region (Hu et al., 1991); by single-round PCR, a 394 bp segment in the first intron of the BZLF-1 gene (Packham et al., 1993); and by single-round PCR, a 246 bp segment bracketing the type 1/2-specifying domain of the EBNA-3C gene in the BamHI E region (Sample et al., 1990). Sequences and coordinates of the primers are shown in Table 1. The EBNA-3C coding region was examined, even though sequence polymorphism therein may not be as marked as in the BamHI K, N and Z regions, because of its potential to allow reliable discrimination between types 1 and 2 (Sample et al., 1990). PCR was performed according to standard procedures. The reaction mixture (50 µl) consisted of each primer at 20 pM, 50 mM MgCl₂, 5 U Taq DNA polymerase and each of the deoxynucleoside triphosphates at 100 mM. PCR was performed for 35 cycles (denaturation at 94 °C for 1 min, annealing at 60 °C (68 °C for the K region primers) for 40 s and extension at 72 °C for 1 min). Before the start of each reaction, the samples were denatured for 5 min at 94 °C; after the last cycle, the extension step was extended by 10 min. Further amplification of BamHI K and N amplicons was carried out, as follows. Two µl of the first-round PCR mix was added to 48 µl second-round PCR mix, which contained the inner primers of the BamHI K amplicon or the hemi-nested anti-sense primer of the BamHI N region; PCR was carried out for 25 cycles for the BamHI K primers (1 min at 94 °C, 40 s at 60 °C, 1 min at 72 °C) and 35 cycles for the BamHI N primers (1 min at 94 °C, 40 s at 62 °C, 2 min at 72 °C).

PCR cloning. Clones were generated from the PCR products derived from tissues with the LigATor cloning kit (R & D Systems). Clones were then transformed into competent Escherichia coli cells, and 14–20 white colonies were picked for analysis.

PCR sequencing. Initial experiments examined the extent of DNA sequence heterogeneity in the EBV-infected cell lines by cycle sequencing. PCR products were purified with GeneClean II and sequenced with the Taq DyeDeoxy Terminator Cycle Sequencing kit and an ABI 373A DNA sequencer (Perkin-Elmer). Both strands from each product were sequenced.
Table 1. Nucleotide sequences and coordinates of primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
<th>5’ end*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI K region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer, sense</td>
<td>5’TGATGGAGCGAGGCGCAAAAAAG</td>
<td>109311</td>
</tr>
<tr>
<td>Outer, anti-sense</td>
<td>5’GAAACCGAAGGAGCAATCTACT</td>
<td>109780</td>
</tr>
<tr>
<td>Inner, sense</td>
<td>5’CGAAAAAGGAGGTTGTTT</td>
<td>109324</td>
</tr>
<tr>
<td>Inner, anti-sense</td>
<td>5’CATCGTCAAAAGCTGACACAG</td>
<td>109756</td>
</tr>
<tr>
<td>BamHI N region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5’GAGAAGGAGAGCAAGGCTA</td>
<td>169382</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’CGCAGCCTTCTTCAATTTCG</td>
<td>169779</td>
</tr>
<tr>
<td>Hemi-nested, sense</td>
<td>5’AAGGCGTTCAGGAGGAGAGA</td>
<td>169394</td>
</tr>
<tr>
<td>BamHI Z region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5’GCCAGGCTGATTTCACAG</td>
<td>102304</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’CACCAGTCCAGATGGTTG</td>
<td>102669</td>
</tr>
<tr>
<td>BamHI E region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5’AGAAGGGGAGGCTGTTGTTG</td>
<td>99939</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’GGCTCGTTTTTGAGCTGAGCC</td>
<td>100091</td>
</tr>
</tbody>
</table>

* Coordinates of primer 5’ ends correspond to the sequence of the B95-8 EBV genome.

EBV genetic diversity

Routine amplification of EBV subgenomic amplicons for each genome region (24 h for changes between P3HR-1 and AG876 (1% similarity between B95-8 by one base substitution, while there were five base substitutions in Namalwa and Daudi sequences, differing from B95-8 by one base substitution, while there were five base substitutions in Namalwa and Daudi sequences, differing from B95-8 by one base substitution, while there were five base substitutions in the type 2 variants.

Again, the extent of variation was small between types: Namalwa and Daudi shared the same sequence, differing from B95-8 by one base substitution, while there were five base substitutions in the type 2 variants.

DNA fragments from the BamHI E and Z regions were not amplified from Raji cells, reflecting deletions in these regions (Rymo et al., 1981). No type-specific differences could be discerned in the fragments amplified from the BamHI N and K regions, but there were variations between lines. For the BamHI N segment, dissimilarities from B95-8 ranged from 2-3% in AG876 to 7-2% in P3HR-1 and Namalwa (Fig. 1C), while for the K segment, dissimilarities from B95-8 ranged from 4-1% in P3HR-1 to 5-9% in Namalwa (Fig. 1D).

Only type-specific size polymorphisms were evident following agarose gel electrophoresis of DNA fragments amplified from the four EBV subgenomic regions (Fig. 2A–D, upper panels). When the PCR products were subjected to SSCP analysis (Fig. 2A–D, lower panels), intra-type mobility shifts were not observed with the BamHI E PCR products (Fig. 2A, lower panel), but were evident between P3HR-1/AR-Ramos and AG876 for the BamHI Z fragment (Fig. 2B, lower panel), and before the N and K products known to bear unique sequences (Fig. 2C, D lower panels). The band positions of the BamHI N and K amplicons were indistinguishable between B95-8, EB176 and EB185, and between P3HR-1 and AW-Ramos (Fig. 2C, D lower panels). There were variations in the intensities of the lower bands for the N fragment (Fig. 2C, lower panel).

**EBV BamHI N and K sequence diversity in clinical specimens**

The data from the cell lines showing that the BamHI N and K segments were more hypervariable than those of E and Z segments (Figs 1 and 2) led us to investigate whether naturally occurring EBV may also exhibit sequence polymorphisms in the N and K regions. To circumvent complete sequencing of the N and K amplicons, we used SSCP to scan for nucleotide
D. Triantos and others

Fig. 1. For legend see facing page.

100066

GTTCAC

B95.8

P3HR-1

AG876

Namalwa

Daudi

102344

GGGACGAAAAAGCTGCCTATAGGATGGGTACCTGA

P3HR-1

AG876

Namalwa

Daudi

102443

GGCAGTCTCTCTACTGGTCAAGAGCTCGTCTTACCTG

P3HR-1

AG876

Namalwa

Daudi

102543

GGGAGCTCTCTACTGGTCAAGAGCTCGTCTTACCTG

P3HR-1

AG876

Namalwa

Daudi

102643

GGCCC

---

P3HR-1

AG876

Namalwa

Daudi

Fig. 1. For legend see facing page.

differences. Differences in SSCP banding patterns between each patient were evident when applied to EBV-positive tongue scrapings of HIV-infected people, with no two samples sharing the same overall pattern, except for lanes 2 and 4 which appear similar (Fig. 3 A). When the SSCP assay was applied to ten HIV-infected patients from whom BamHI N amplicons could be obtained from both tongue scrapings and CD45-enriched PBCs (Fig. 3 B), within-person identity of banding patterns was certain in one patient (no. 10), and possible in four (nos 2, 5, 7 and 8); for the rest, within-person differences were clearly apparent. To examine if such intra-host differences applied to other body sites, the same procedure was used to examine BamHI N amplicons derived from whole saliva, tongue and parotid saliva of another HIV-infected patient (no. 11) in whom EBV DNA could be amplified from all three compartments; as Fig. 3 (C) (left panel) shows, the patterns were distinguishable from each other. The procedure was used further to investigate if, over a period of time, an EBV variant shed into the oral cavity might change. As Fig. 3 (C) (right panel) shows for patient 12, for whom whole saliva was obtained regularly over a 12-month period, the same pattern of mobility shift was seen at each sampling. The figure also shows that over the same period the band positions from the saliva of patient 13, who was a homosexual partner of patient 12, were identical to those from patient 12, although there were some variations in band intensities.

**EBV BamHI K sequence diversity in lesion specimens**

We then studied sequence polymorphism in the BamHI K amplicons recovered from seven lesions previously identified to be EBV DNA-positive. SSCP analysis showed considerable inter-lesion variability (Fig. 4). This was confirmed by DNA sequence analysis. Fig. 5 shows a comparison between cell lines and between the seven lesions of the amino acid sequences predicted from the sequences of the amplified BamHI K segment. None of the lesion sequences was identical to any other or to any cell line.

To examine, further, whether different variants could be carried in the same lesion, sequence polymorphism was examined of cloned BamHI K amplicons derived from selected OHL, NPC and oral ulcer specimens. Fig. 6 shows a result obtained by SSCP analysis of OHL and NPC tissue; while at least four SSCP banding patterns (designated I–IV) were discernible in the OHL lesion, all the bands of the NPC specimen were identical.

Similar SSCP analyses (not shown) were conducted of 20 clones derived from two oral ulcer lesions, one from an HIV-infected person (lesion no. 5 in Fig. 4) and one from a presumed
HIV-seronegative person with Sweet's syndrome (lesion no. 6 in Fig. 4). For lesion 5, one of the 20 clones yielded two non-synonymous base changes compared to the dominant sequence represented in the other 19 clones; one of the 20 clones of lesion 6 had four non-synonymous base changes relative to the dominant sequence (Fig. 5).

Discussion

EBV cannot be propagated directly in vitro. In order to investigate the diversity of naturally occurring EBV, the conventional approach has been to transform B cells into permanent lymphoblastoid cell lines, after which viral DNA or protein extracts are submitted to various differentiation procedures such as DNA restriction-fragment length polymorphism (RFLP) assays (Lung et al., 1988) or Western blot analysis of EBNA size polymorphisms ('Ebnotyping') (Gratama et al., 1994). A limitation of this approach is the potential for selection against EBV strains that transform lymphocytes either poorly, such as the type B viruses (Rickinson et al., 1987), or not at all, such as EBNA-2-deletion variants (Sixbey et al., 1991). Alternatively, EBV DNA sequences are amplified directly from a given tissue or body fluid, by procedures such as type-specific PCR (Buisson et al., 1994; Aitken et al., 1994; Menin et al., 1996), post-PCR type-specific oligonucleotide probing (Sixbey et al., 1989; Yao et al., 1991; Apolloni & Sculley, 1994), gel-electrophoretic analysis of size polymorphisms of PCR products (Lin et al., 1993; Miller et al., 1994;
D. Triantos and others

Fig. 2. Comparison of PCR products amplified from four regions of the EBV genome carried in various lymphoid cell lines. PCR products were (upper panels) stained with ethidium bromide after agarose gel electrophoresis, or (lower panels) stained with SYBR-GREEN II after non-denaturing PAGE conducted after heat denaturation of the PCR products. (A) BamHI E; (B) BamHI Z; (C) BamHI N; and (D) BamHI K. Lanes are labelled as follows: M, molecular mass markers; 1, B95-8; 2, EB176; 3, EB185; 4, P3HR-1; 5, AW-Ramos; 6, AG876; 7, Namalwa; 8, Daudi; 9, Raji; 10, water control.

Falk et al., 1995; Chen et al., 1996) and post-PCR RFLP assays (Bhatia et al., 1996). The discriminatory ranges of these procedures are finite, however, so sequencing is often required to examine further heterogeneity within the amplified fragments (Buisson et al., 1994; Aitken et al., 1994; Miller et al., 1994; Sandvej et al., 1994; Busson et al., 1995; Bhatia et al., 1996; Gutiérrez et al., 1997).

The nucleotide sequence variability in the EBNA-3C gene segments (Fig. 1 A) segregated genotypically, as expected (Sample et al., 1990); the degree of dissimilarity was up to 4% in type 1 viruses (B95-8, Namalwa and Daudi) and < 1% in type 2 viruses (P3HR-1 and AG876). The variability in the BZLF-1 gene fragment also segregated along genotypic lines (Fig. 1B); the range of sequence dissimilarity between type 1 viruses was < 1%, and between type 2 viruses was < 2%.

We found more substantial heterogeneity in the region that encompasses and is upstream of the LMP-1 start codon in the BamHI N region (Fig. 1C), and in the C-terminal unique domain of the EBNA-1 ORF in the BamHI K region (Fig. 1D). The nucleotide differences were not genotype-related. Thus, in the BamHI N fragment the range of sequence dissimilarities (when compared to the prototypic B95-8) was 2–7%, and that in the BamHI K fragment was 4–6%. Detailed sequencing studies have identified potential variability in the BamHI N region (Hu et al., 1991) and, in particular, the heterogeneity of the LMP-1 ORF has been verified in a broad range of EBV-associated lesions (Miller et al., 1994; Sandvej et al., 1994; Palefsky et al., 1996). Sequence heterogeneity in other subgenomic regions, such as the C-terminal domain of the EBNA-1 gene, has also been alluded to (Wrightham, 1995; Snudden et al., 1995; Bhatia et al., 1996; Gutiérrez et al., 1997).

The findings of Gutiérrez et al. (1997) are particularly interesting, in that the cognate amino acid sequences we identified (Fig. 5) can be classified into three of their five subtypes, which were based on a stretch of amino acid sequence corresponding to positions 469–530 of the C
EBV genetic diversity

Fig. 3. (A) Heterogeneity in SSCP banding patterns produced by PCR products amplified from the EBV BamHI N region (upper panel) and K region (lower panel) in tongue scrapes of HIV-infected patients. Different patients are represented by different lanes. (B) Comparison of BamHI N SSCP bands derived from blood (b) and tongue (t) specimens of ten HIV-infected patients (patients 1–10). (C) Left panel: BamHI N SSCP banding patterns derived from whole saliva (s), tongue (t) and parotid saliva (p) of an HIV-infected patient (patient 11). Right panel: BamHI N SSCP banding patterns derived from whole saliva of HIV-positive homosexual partners (patients 12 and 13) taken at 3-monthly intervals. Numbers below denote times of sampling (months).

Fig. 4. BamHI K SSCP banding patterns derived from tissue biopsy specimens. Lanes are labelled as follows: 1 and 2, lung (bronchogenic carcinoma); 3 and 4, skin (squamous cell carcinoma); 5, oral mucosa (mouth ulcer from HIV-infected patient); 6, oral mucosa (Sweet’s syndrome); 7, post-nasal space mucosa (NPC).

terminus of EBNA-1. Thus, the sequences from B95-8 and from lesion 4 fall into the P-ala subtype, those from Daudi and lesion 7 may be classified as P-thr, and the others (P3HR-1, AG876, Namalwa, Raji and lesions 1–3, 5 and 6) may be classified as V-leu. However, it is apparent from Fig. 5 that study of the amino acid sequence between positions 469–530 does not permit the full extent of EBV diversity to be appreciated. In particular, for P-thr- and V-leu-like variants, there are motifs between positions 587–596 that allow for further differentiation. Furthermore, point mutations elsewhere permit even finer intra-subtype discrimination to be made.

The hypervariability in the BamHI K and N regions is thus sufficiently wide to be used as an index of EBV diversity. We find that the SSCP procedure provides a satisfactory alternative for evaluating sequence polymorphisms in the two regions (Figs 3 and 4). Its non-radioisotopic nature potentially allows large numbers of EBV-infected tissues and body fluids to be screened for EBV genomic variation. The procedure also provides a simple means of examining EBV clonality; the findings in Fig. 6 confirm the monoclonality of EBV in NPC, but not OHL (Miller et al., 1994).

Previous studies showed that an individual host, particularly one who is HIV-infected, can shed different EBV variants from different body sites (Sixbey et al., 1989; Oosterveer et al., 1993; Gratama et al., 1994; Apolloni & Sculley, 1994; Yao et al., 1996). There is also growing evidence that strictly epitheliotropic EBV strains exist (Chen et al., 1996; Gutiérrez et al., 1997). In all these studies, the shedding sites examined tended to be disparate. Here, we demonstrate intra-host diversity in proximate oral compartments (Fig. 3C, patient 11), suggesting that different variants can occupy specific niches. Whether such occupation reflects selective tropism of EBV is unknown. Identification of the same EBV variant from the mouths of patients 11 and 12 over a protracted period (Fig. 3C) may be due to that variant being persistently shed into their oral cavities from unidentified sites or to persistent reinfection from one partner to the other.

Our study of BamHI K clones derived from two oral ulcer lesions (Figs 4 and 5) shows that a given non-OHL lesion site can carry more than one EBV variant. It is not clear whether the minority sequence evolved from the dominant one in each lesion, since the number of point mutations in the minority sequence was small, and the changes did not alter subtype-specific motifs (Gutiérrez et al., 1997). However, since inter-lesion sequence variation can be very restricted (there were only three base changes between lesions 5 and 6), it remains a possibility that in each lesion the majority and minority sequences reflect dual infection by independent variants. All these sequences are related to the V-leu subtype, first identified in Burkitt’s lymphoma tissues (Snudden et al., 1995). The
Fig. 5. Comparison of cognate amino acid sequences from positions 466–596 of EBNA-1 protein of EBV in cell lines and tissue biopsies. Numbers to the right correspond to lane numbers in Fig. 4. Si and 6i denote the sequences of single clones of 20 clones derived from BamHI K amplicons of specimens 5 and 6; the sequences of the other 19 clones from each specimen were identical to one another and to the sequence obtained by direct PCR sequencing.

Fig. 6. SSCP banding patterns of clones of BamHI N PCR products derived from an OHL (A) and an NPC (B) biopsy specimen. In (B), the middle band in the 4th lane from the right was too faint to be reproduced in the figure. I–IV indicate four distinct banding patterns.

We are grateful to Drs S. Hinrichsen and J. Epstein for the provision of study specimens. This work was supported by the UK Medical Research Council (grant SPG 9009231) and an award to D.T. by the State Scholarships Foundation, Republic of Greece.

References


finding that EBV variants characterized from lesions 1, 2 and 3 (Fig. 4) are also closely related to V-leu suggests that this subtype is not exclusively lymphotropic, since these tissue specimens were not lymphoid in origin.

Variation in certain genomic segments of naturally occurring EBV therefore can be extensive, extending beyond groupings based on dichotomous genotypic differences, the number of internal nucleotide repeats, and DNA or amino acid sequence motifs. Given the relative immutability of herpes viruses (Smith & Inglis, 1987), the existence of hypermutable loci in EBV is unexpected, and further studies into how genetic microheterogeneity supports the survival of this virus should be rewarding.


Received 6 May 1998; Accepted 21 July 1998